



Enhanced *ex vivo* expansion of adult mesenchymal stem cells by fetal mesenchymal stem cell ECM[☆]



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ABSTRACT

Large-scale expansion of highly functional adult human mesenchymal stem cells (aMSCs) remains technologically challenging as aMSCs lose self renewal capacity and multipotency during traditional long-term culture and their quality/quantity declines with donor age and disease. Identification of culture conditions enabling prolonged expansion and rejuvenation would have dramatic impact in regenerative medicine. aMSC-derived decellularized extracellular matrix (ECM) has been shown to provide such microenvironment which promotes MSC self renewal and “stemness”. Since previous studies have demonstrated superior proliferation and osteogenic potential of human fetal MSCs (fMSCs), we hypothesize that their ECM may promote expansion of clinically relevant aMSCs. We demonstrated that aMSCs were more proliferative ($\sim 1.6\times$) on fMSC-derived ECM than aMSC-derived ECMs and traditional tissue culture wares (TCPS). These aMSCs were smaller and more uniform in size (median \pm interquartile range: $15.5 \pm 4.1 \mu\text{m}$ versus $17.2 \pm 5.0 \mu\text{m}$ and $15.5 \pm 4.1 \mu\text{m}$ for aMSC ECM and TCPS respectively), exhibited the necessary biomarker signatures, and stained positive for osteogenic, adipogenic and chondrogenic expressions; indications that they maintained multipotency during culture. Furthermore, fMSC ECM improved the proliferation ($\sim 2.2\times$), size ($19.6 \pm 11.9 \mu\text{m}$ vs $30.2 \pm 14.5 \mu\text{m}$) and differentiation potential in late-passaged aMSCs compared to TCPS. In conclusion, we have established fMSC ECM as a promising cell culture platform for *ex vivo* expansion of aMSCs.

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1. Introduction

Human mesenchymal stem cells (MSCs) are bone marrow-derived multipotent cells which are involved in tissue regeneration throughout a person's life. Due to their self renewal capacity, differentiation and therapeutic potential these cells are also of great clinical interest. Currently researchers are exploring the utility of

these cells for the treatment of many acute, chronic, and degenerative diseases including Crohn's disease, multiple sclerosis, graft-versus-host disease, type 1 diabetes, bone fractures, cartilage defects, etc [1–3]. However, a major bottleneck in clinical use of adult mesenchymal stem cells (aMSCs) is their low prevalence which requires prolonged *ex vivo* expansion to achieve a useful number of cells; expansion which results in the cells' loss of many of their useful stem cell properties.

The standard method of isolating and culturing aMSCs is through plastic adherence of mononuclear cells from bone marrow (BM) aspirate. When cultured under appropriate conditions the aMSCs adhere and proliferate while contaminating cell types perish resulting in a relatively pure population of progenitors after 2–3

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passages. However, the percentage of progenitors collected from BM aspirate is small. Using colony forming unit assays (CFUs), the prevalence of aMSCs in BM aspirates has been measured to be between 0.001 and 0.01% of the nucleated cells within the sample [4–6]. This means these cells must undergo prolonged expansion in order to obtain clinically relevant cell numbers. For example, a dose of 1–2 millions cells per kg of body weight or more (about 70–140 million cells for average human of weight 70 kg) is required for intravenous infusion and treatment of a wide variety of conditions such as graft-vs-host disease (GVHD), bone and cartilage defects, myocardial infarction and autoimmune diseases [2,7].

Currently large-scale expansion of highly functional aMSCs is an unaddressed challenge as MSCs undergo drastic phenotypic changes during prolonged culture on tissue culture polystyrene (TCPS). Specifically, the proliferation capacity of the cells decreases, the self renewal capacity of the cells diminishes, and the cells often begin to express behavior of cells with a committed lineage or reach senescence. Furthermore, MSC quantity/quality declines with donor age and disease, thus it is indeed challenging to expand sufficient numbers for treatments in older donors who are typically the patients in need of such therapy. These limitations clearly illustrate the need to develop cell culture conditions which enable prolonged expansion of the stem cells without loss of their stem cell characteristics. One type of cell culture substrate which has recently shows promise in maintaining aMSC phenotype during *ex vivo* expansion is decellularized extracellular matrix (ECM). During *ex vivo* culture cells produce their own ECM. Through appropriate processing the matrix can be decellularized, meaning the cells and cellular debris are removed leaving behind only the extracellular components. Previously, Lin et al. showed that decellularized aMSC ECM improved the proliferation, attachment, spread, migration, and multi-lineage differentiation capacity of aMSCs in comparison to cells grown in collagen type I [8]. Lai and co-workers demonstrated the expansion of human MSCs on their ECM strongly promoted cell proliferation, maintained stem cell properties, and resulted in low levels of reactive oxygen species being produced [9]. In contrast, bone formation capacity of cells expanded on tissue culture plastic was dramatically diminished after 6–7 passages. Also, Sun et al. has shown that culturing freshly isolated human bone marrow mononuclear cells on stromal cell-derived extracellular matrix enhances the formation of colonies comprised of either osteoblast-like, fibroblast-like, or adipocyte-like cells [10]. In addition, they also showed that culturing late-passage MSCs on fresh ECM recovered or at least retained the desired properties in these older cells. These recent studies clearly illustrated the potent ability of decellularized ECM to maintain, and in some cases recover desirable aMSC behavior. The cellular mechanisms which result in loss of aMSC “stemness” during prolonged expansion remain unclear; however, these previous works illustrate that ECM may be an important factor. One could envision that over multiple passages compositional changes in ECM may occur which results in an extracellular environment lacking certain cues necessary for the maintenance of aMSC phenotype, and by culturing the stem cells on a more biologically complete matrix the desired cell behaviors can be maintained/recovered. Plating of cells on ECM isolates such as collagen or laminin or synthetic substrates would likely lack the appropriate cues. Thus, MSC ECM may be a more biologically complete substrate and aid in the maintenance of the desired aMSC phenotype.

In this report, we investigate the use of fMSC to generate ECM-based substrates for *ex vivo* expansion of aMSCs. There are several potential reasons why fMSC ECM may offer a superior cell growth substrate compared to aMSC ECM and other tissue-derived MSC ECMs. In previous work, Zhang and co-workers have illustrated higher proliferative and osteogenic capacity of fMSC compared to aMSCs, human umbilical cord MSCs, and human adult adipose

tissue MSCs [11]. The higher proliferation capacity of the cells may indicate greater amounts of ECM production meaning more ECM is produced making these cells a more productive source of ECM. Also, since the quality and quantity of MSCs has been documented to decline with age, less ECM will be collected from cell isolated from an adult bone marrow sample and the ECM which is produced will likely be of less consistent quality. The most primitive and proliferative BM MSCs are from fetal bone marrow in the constant state of tissue development indicating that they may be the best source of ECM for the maintenance of aMSC phenotype. Furthermore, the process of decellularization destroys the cells that are used to produce them; this implies loss of a substantial amount of the expanded aMSCs to produce the matrix; cells which could instead be used for therapeutics. Here, we perform 2 studies to evaluate the utility of fMSC ECM as a cell culture substrate for the expansion of highly functional aMSCs. First, we assess the (1) proliferation, (2) cell size distribution (as indicators of “stemness” [12,13]), (3) immunophenotypic expressions and (4) differentiation capacities of expanded aMSCs seeded on fMSC ECMs in comparison to substrates such as standard TCPS and ECMs regenerated from aMSCs and human neonatal dermal fibroblasts (nHDF). In the second study, we explore the effects of fMSC ECMs on late-passaged aMSCs versus TCPS in terms of the 4 parameters as indicated earlier for the first study. We also quantify the amount of ECM generated from the various cell types to understand their scalability potentials for large-scale therapeutic applications.

2. Materials and methods

2.1. Cell culture and maintenance of fMSC, nHDF and aMSC

Human fetal mesenchymal stem cells (fMSC) were isolated from fetal femurs after clinically indicated termination of pregnancy as previously described [11]. All human tissue collection for research purposes was approved by the Domain Specific Review Board of National University Hospital Singapore, in compliance with international guidelines regarding the use of fetal tissue for research. In all cases, patients gave separate written consent for the use of the collected tissue. Briefly, single-cell suspensions were prepared by flushing the BM cells out of femurs using a 22-gauge needle, passing through a 70- μ m cell strainer (BD Biosciences, San Diego, CA), and plating on Nuclon™ Delta T75 culture flasks (Thermo Scientific Nunc, Rochester, NY) at 10^6 cells/ml. Adherent spindle-shaped cells were recovered from the primary culture after 4–7 days. Non-adherent cells were removed with initial medium changes every 2–3 days. fMSCs were isolated and cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA) for fMSC supplemented with 10% MSC qualified fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (Life Technologies, Carlsbad, CA), hereafter referred to as D10 medium.

Passage 2 human adult mesenchymal stem cells (aMSCs) were obtained from Lonza (Basel, Switzerland) and cultured according to manufacturers' instructions. aMSC were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% MSC qualified FBS and 1% penicillin/streptomycin (Life Technologies), hereafter referred to as DLG10 medium.

Passage 1 human neonatal dermal fibroblasts (nHDF, ATCC® PCS-201-010™) were obtained from ATCC (Manassas, VA) and expanded according to manufacturers' instructions. Neonatal dermal fibroblasts were cultured and expanded to passage 3 in Fibroblast Basal Medium (ATCC® PCS-201-030™) supplemented with Fibroblast Growth Kit-Low serum (ATCC® PCS-201-041™) and 1% penicillin/streptomycin (Life Technologies).

Cells were plated in Nuclon™ Delta T-75 flasks at 500 cells/cm², incubated at 37 °C and 5% CO₂, and medium was changed every 2–3 days. Passage 2 cultures were expanded up to a maximum of 70% confluence, detached with 1 × TrypLE Express (Life Technologies, Carlsbad, CA) unless otherwise stated and cryopreserved in appropriate growth medium with 10% DMSO (Sigma Aldrich, St Louis, MO) and 30% MSC qualified FBS to create a stock of cells. For cell experiments, cells from the stock were thawed and cultured according to the above protocol. Passages 4 were used in cell experiments to screen and characterize the various substrates. D10 medium as described previously was used in all experiments.

2.2. Preparation of decellularized ECM, fibronectin-coated substrates and conditioned medium

Decellularized ECM substrates were prepared with modifications from a previous protocol [14] by plating cells in 6-well plates at 1000 cells/cm² and cultured for 14 days with fresh media replacement every 2–3 days (Fig. 1a). A final concentration of 50 μ M ascorbic acid (Sigma Aldrich, St Louis, MO) was added to the media for the

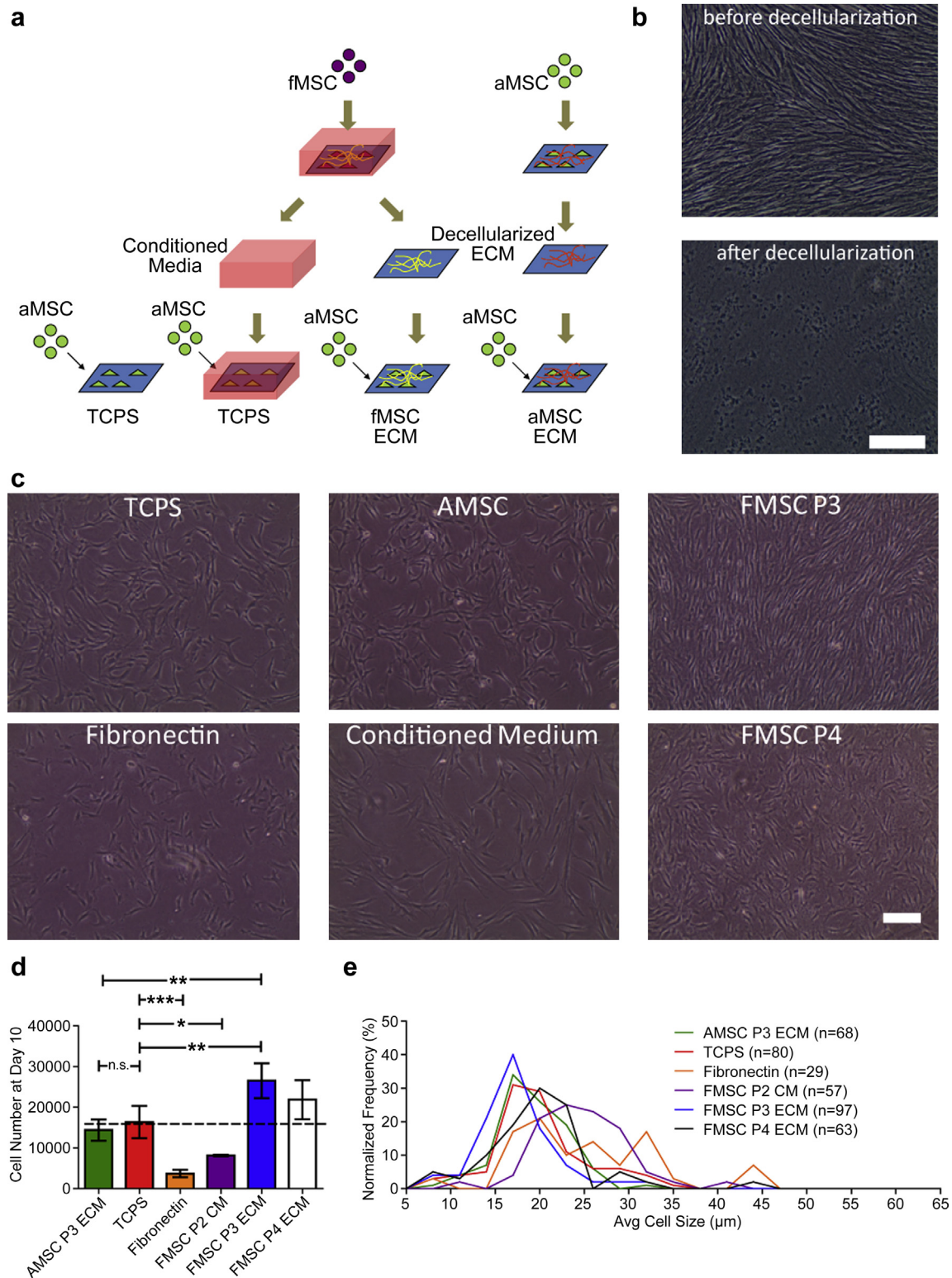


Fig. 1. (a) Schematic of decellularized ECM study. MSCs are plated on TCPS and cultured to generate extracellular matrices. The MSCs are decellularized to expose the layer of ECM for proliferation studies with aMSCs. In addition, media is collected from the fMSC cultures for the conditioned media (CM) condition. To assess proliferation, aMSCs of passage 4 were seeded on TCPS controls and decellularized matrices, cultured for 10 days before detachment to evaluate cell number and cell size. (b) Micrograph showing cell monolayer before and after the decellularization process. The decellularization protocol successfully remove the cells leaving a layer of extracellular matrices. Scale Bar = 200 μm . (c) Micrographs showing adult mesenchymal stem cells cultured on the various conditions. Qualitatively, aMSCs cultured on decellularized matrices are morphologically similar to cells cultured on TCPS. Scale Bar = 200 μm . (d) Cell yield ($n = 3$) and (e) size distribution of aMSCs cultured under various conditions. Statistical analysis with 1-way ANOVA with Tukey's Multiple Comparison Test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

last 7–8 days of culture to increase the production of ECM. Passage 3 cells were used to produce the matrices (Fig. 1b) unless otherwise stated. The cells were rinsed with PBS and treated with PBS containing 0.5% Triton X-100 (Sigma Aldrich, St Louis, MO) and 20 mM NH₄OH (Sigma Aldrich, St Louis, MO) for 5 min at 37 °C. ECM remaining in the wells were rinsed with PBS and treated with DNase (Sigma, ≥100 units/ml PBS) for 1 h at 37 °C. Following additional PBS rinsing, plates were allowed to dry within a sterile biosafety cabinet. ECM-containing plates were stored at 4 °C in the dark in a closed sterile container or sterile sealable bag until use within 1 month.

Fibronectin-coated substrates were prepared according to Marcantonio et al. with slight modifications [5]. Briefly, fibronectin (Sigma, St. Louis, MO) was adsorbed to tissue culture substrates by incubating substrates with a 10 µg/mL solution in PBS for 2 h at room temperature, rinsing 3× with PBS, then blocking for 1 h at room temperature with 1% bovine serum albumin (Sigma, St. Louis, MO), and rinsing 3× again with PBS. Fibronectin coated culture wares were stored for 2–4 weeks at –8 °C in a closed sterile container or sterile sealable bag.

To generate conditioned media, fMSCs were plated at a density of 1000 cells/cm² with D10 medium. On the following day, the medium was replaced with DLG10 medium for conditioning. The conditioned DLG10 medium was collected every 24 h until the cells reached confluence, sterile filtered with a 0.22 µm syringe filter and stored at –20 °C until use.

2.3. Cell proliferation study

aMSC were inoculated at 100 cells/cm² on the decellularized fMSC ECM and cultured for 10 days with fresh media change every 3 days. Cells were detached using TrypLE Express or Accutase (Sigma Aldrich, St Louis, MO), samples counterstained with trypan blue (0.4%) (Sigma Aldrich, St Louis, MO) and counted using a hemocytometer. Images were also acquired for cell size distribution and analyzed using ImageJ image processing software from NIH, Bethesda, MD.

To perform cell size distribution analysis, cells monodispersed by trypsinization was examined by cellular image analysis using ImageJ. The major and minor axes of the cells were measured, averaged and presented as the cell sizes. Their normalized (relative) frequency distributions, normalized against the total number of cells counted per condition, were plotted against the average cell size (determined by averaging the major and minor axes) in 3 µm bins. For quantification of the cell size distributions, the median, 25% percentile, 75% percentile and the interquartile range (IQR, a measure of statistical dispersion equal to the difference between the upper 75% and lower 25% quartiles) of the normalized frequency distributions were determined using GraphPad Prism 5 software (La Jolla, CA).

Cumulative population doubling level at each subcultivation was calculated from the cell count by using the equation [15]:

$$N_H/N_I = 2^X,$$

or

$$X = [\log_{10}(N_H) - \log_{10}(N_I)]/\log_{10}(2),$$

where N_I = inoculum number, N_H = cell harvest number, and X = population doublings. The population doubling increase that was calculated was then added to the previous population doubling level (PDL), to yield the cumulative population doubling level.

2.4. Plating efficiency study

MSCs were harvested by trypsinization and washed twice with a serum-free medium, and 5×10^4 aMSCs were inoculated per well of 2-well LabTek chambered glass well slides (Thermo Scientific Nunc, Rochester, NY) prepared with the ECM substrates. For TCPS comparison, cells were seeded on 12 wells Nunc Nuclon™ Delta plates, which had an equivalent surface area (4 cm²) to the well slides. After incubation for 2 h, the wells were rinsed twice to remove unattached cells. The attached cells were fixed using 4% paraformaldehyde (PFA) (Sigma Aldrich, St Louis, MO) for 15 min and stained with DAPI (5 µg/ml, Life Technologies, Carlsbad, CA) for 20 min. Mosaic images of the entire wells were acquired by epi-fluorescence microscopy using a customized fast-rastering Leica DMI 6000B microscope (ImageIQ, Cleveland, OH). Cells were counted using the “analyze particles” plugin in ImageJ image processing software (NIH, Bethesda, MD). Plating efficiencies (PE) measured how efficient the cells adhered to the substrates and were calculated as a fraction:

$$PE = N_A/N_P$$

where N_A = adhered cell number and N_P = plated cell number. A PE of 1 implied that all the plated cells adhered while on the other end of the spectrum, a PE of 0 indicated no adhesion.

2.5. Immunophenotyping

To minimize the variations, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed minimal criteria to define hMSC populations. These include: (i) hMSCs must be plastic-adherent when maintained in classical culture conditions; (ii) hMSCs must express high levels (≥95% positive) of CD105, CD73, and CD90 and lack expression. (≤2% positive) of CD45, CD34, CD14, or CD11b, CD79α or CD19, and HLA-DR (unless stimulated by

interferon-γ) surface molecules; (iii) hMSCs must differentiate into osteoblasts, adipocytes, and chondroblasts under specific *in vitro* differentiation conditions [2,16].

The various biomarkers and negative control isotypes used for screening were purchased from BD Biosciences (San Jose, CA) and listed in Supplementary Table S1. Some of the markers were not tested on the later passaged cells due to their limited yield. CD14 or 11b expression was not also tested. Briefly, cells were rinsed with PBS, detached with accutase and incubated at 37 °C in incubator for 15 min to detach cells. Cells were washed, samples counterstained with trypan blue (0.4%) (Sigma Aldrich, St Louis, MO) and counted using a hemocytometer. The rest of the cells were divided appropriately into tubes containing the antibodies, mixed well and incubated for 1 h in the dark. The labeled cells were washed with PBS before fixation in 1% PFA, and screened using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) within 1 week.

2.6. Differentiation assays

Osteogenesis induction was performed with StemPro(R) Osteogenesis Differentiation Kit (Gibco, Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Briefly, aMSCs were plated on various substrates at 5000 cells/cm² and cultured in DLG10 medium for 48 h before changing to differentiation media. The cells were subsequently cultured for 13–14 more days with media change every 3 days before detection for differentiation. Osteoblast differentiation was detected by alkaline phosphatase (AP) expression using SIGMAFAST™ BCIP/NBT substrate (Sigma Aldrich, St Louis, MO). AP activity is an indication of successful differentiation of MSC into osteoblasts. Undifferentiated MSCs show weak alkaline phosphatase (AP) activity, whereas differentiated osteoblasts feature very high AP and stain blue violet using BCIP/NBT as a substrate. Cell were washed with PBS and fixed with pre-cooled methanol for 5 min at –20 °C. This was followed by rinsing with mQ-H₂O before incubation with dissolved SIGMAFAST™ BCIP®/NBT substrate for 10 min under slow agitation on a plate shaker at room temperature. The stain solution was removed and the cells were washed with mQ-H₂O and imaged immediately while kept moistened.

Adipogenesis induction was performed with StemPro(R) Adipogenesis Differentiation Kit (Gibco, Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Briefly, aMSCs were plated on various substrates at 5000 cells/cm² and cultured in DLG10 medium for 48 h before changing to differentiation media. The cells were subsequently cultured for 13–14 more days with media change every 3 days before detection for differentiation. Adipocytes were detected by their large numbers of intracellular lipid vesicles using Oil Red O staining (Sigma Aldrich, St Louis, MO) and identified by their bright red color. Cells were washed with PBS and fixed with neutral buffered formalin (10%) for 30 min at room temperature. This was followed by rinsing with mQ-H₂O before incubation with 60% isopropanol to cover the cell monolayer for 5 min at room temperature. The isopropanol was replaced with 0.3% Oil Red O in isopropanol and incubated for 20 min under slow agitation on a plate shaker at room temperature. The cells were washed with mQ-H₂O and imaged immediately while kept moistened.

Chondrogenic differentiation was done using StemPro(R) Chondrogenesis Differentiation Kit (Gibco, Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Briefly, aMSCs were plated in non-adherent u-bottom 96-well plates at 100,000 cells/well to generate spheroids and cultured in DLG10 medium for 48 h before a change to differentiation media. The cells were subsequently cultured for 13–14 more days with media change every 3 days before detection for differentiation. Chondrogenic differentiation of MSC in 3D spheroid culture results in formation of cartilage with proteoglycan aggregate. Aggregate was detected with Alcian Blue (Sigma Aldrich, St Louis, MO), a dark-blue copper-containing dye. Spheroids were rinsed with PBS before fixation with 10% neutral buffered formalin for 60 min at room temperature. Following thorough rinsing with PBS to remove the fixatives, the spheroids were stained in 1% Alcian Blue solution in 0.1N HCl (prepared from Alcian Blue-8GX, Sigma Aldrich, St Louis, MO) and agitated slowly on a plate shaker for 30 min at room temperature. The spheroids were washed thoroughly with 0.1N HCl to remove the Alcian Blue stain and imaged immediately while kept moistened.

2.7. Image quantification of differentiation expression levels

The expression level in each image was defined by the intensity density of the stain in the image, which is the product of area and mean intensity of the stains in the images. This was determined using ImageJ by adopting the methods from their online example provided to quantify stained liver tissues (<http://rsbweb.nih.gov/ij/docs/examples/stained-sections/index.html>). Briefly, there were five steps to obtaining the quantification:

- (1) converting the color images to grayscale images of individual red, green and blue channels,
- (2) determining the channel with the best separation (BCIP NBT and Oil Red O – green channel; Alcian Blue – red channel)
- (3) isolating the stained areas using thresholding and converting the thresholded portion of the image into a selection mask using the ‘create selection’ function
- (4) transferring the selection mask back to the original color image

- (5) measuring the intensity density of the selection in the image using the 'measure' function

The ImageJ macro for the BCIP NBT expression quantification is provided in the supplementary materials as an example (Supplementary Fig. S1).

2.8. Late passage study

In the late passage studies to assess if fMSC ECM substrates could rejuvenate aged aMSCs (Schematic in Fig. 5a), Passage 6 cells previously cultured on TCPS were seeded on fMSC ECM substrates at $100/\text{cm}^2$ and cultured in similar conditions for 3 passages as stated above. They are then assessed for cell proliferation, cell size, differentiation capabilities and MSC biomarker expression as described in the earlier sub-sections.

2.9. Quantification of ECM yield per cell plated

Decellularized ECM substrates were prepared as described in Section 2.2. The substrates were mechanically detached in deionized water using cell scrapers (SPL Life Sciences, South Korea) and weighing them in 1.5 ml eppendorf tubes (pre-weighed and post-dried after drying in a 70°C oven overnight). The weight of the ECM was normalized to the number of cells plated instead of the number of cells at the time of decellularization since it was not possible to determine the latter number due to the destructive nature of the decellularization protocol. In addition, from a scalability perspective, it might be more of interest to determine how much ECM could be generated from the number of cells plated instead.

2.10. Statistical analysis

All data are presented as mean \pm standard deviation calculated, with $n = 3$ replicates or otherwise stated, depending on the experiments. Using GraphPad Prism 5 software, multiple means were compared with either an unpaired t -test or an analysis of variance (ANOVA) test to determine if the means were statistically different. If ANOVA indicated statistical difference, then the Tukey–Kramer Honestly Significant Difference Test was used to determine which of the pairs of means were statistically different. Pairs of means are marked with either (ns) meaning the means or not statistically different or asterisks (*) if the means are significantly different. The number of asterisks indicates the P -value: $p \leq 0.05$ for *, $p \leq 0.01$ for **, and $p \leq 0.001$ for ***.

3. Results

3.1. Effect of proliferation of aMSCs on various substrates

We first started by screening various substrates and conditions, specifically, conditioned medium using expanded passage 4 aMSCs (Fig. 1a). Passage 3 cells were used to prepare the decellularized matrices unless otherwise stated. They were successfully decellularized as visualized by light microscopy (Fig. 1b). The reason for the choice of passage 3 was to maximize the number of cells while ensuring the cells still maintain their undifferentiated phenotypes.

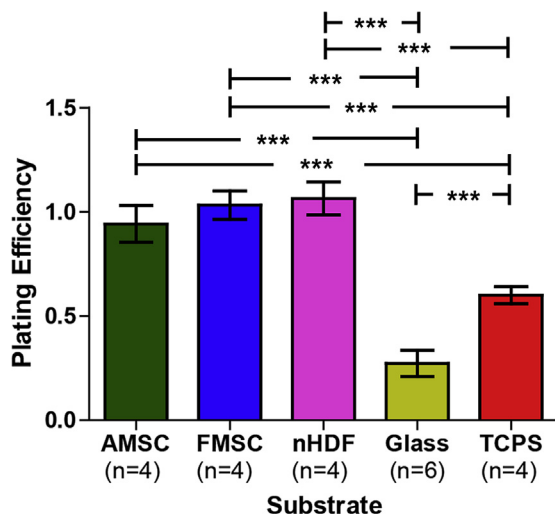


Fig. 2. Plating Efficiency of expanded adult mesenchymal stem cells. Cells were plated at 5.0×10^4 per well and incubated for 2 h before rinsing to fixation. Fixed cells were stained with DAPI (5 $\mu\text{g}/\text{ml}$) for 15 min, imaged and counted using ImageJ. Statistical analysis using 1-way ANOVA with Tukey's Multiple Comparison Test; *** $p \leq 0.001$.

Furthermore, early passage BM cultures contained contaminated non-MSCs of mostly hematopoietic nature that usually cleared up within the 2 passages. It had also been shown by Binato and co-workers that MSCs retain their chromosome stability up to passage 4 [7]. Morphologically, aMSCs cultured on fMSC ECM retained spindle-shaped morphology (Fig. 1c) but qualitatively more in numbers. They also appeared more aligned, probably due to the orientation of the fMSC ECM. aMSCs cultured on fMSC P3 ECM resulted in significantly higher growth than on TCPS as indicated by the cell yield at day 10 (Fig. 1d). On average, the cell number at the end of day 10 culture on fMSC ECM is about $1.6\times$ more than their TCPS counterpart ($n = 3$, $p \leq 0.01$). In addition, the passage/age of the cells used for generating the matrices appear to affect the quality of the ECM as P4 fMSC ECM were less effective compared to P3 fMSC ECM. Growth on P4 ECM was also higher (about $1.3\times$) than that of TCPS ($n = 3$, $p > 0.05$) but lower than culture on P3 ECMs. aMSCs cultured on fMSC ECMs were also more proliferative (about $1.8\times$) than their counterparts on aMSC ECMs. Interestingly here, we did not observe any significant difference ($p > 0.05$) between aMSC yield on aMSC ECMs in comparison to TCPS.

From the cell size distribution data (Fig. 1e and Table 1) in terms of median and interquartile range (IQR), we observed that aMSCs cultured on P3 fMSC ECM (median \pm IQR: $15.5 \pm 4.1 \mu\text{m}$) have slightly smaller and more uniform sizes than TCPS ($17.5 \pm 4.4 \mu\text{m}$) and others (e.g. aMSC ECM: $17.2 \pm 5.0 \mu\text{m}$; P4 fMSC ECM: $18.5 \pm 5.3 \mu\text{m}$; Fibronectin: $22.4 \pm 11.5 \mu\text{m}$). It has been demonstrated that smaller MSCs share similar characteristics as early passage multi-potent MSCs and may be more "stemness" [12,13]. Thus the result here may suggest that culturing aMSCs on the fMSC ECMs may help the adult cells to retain their "stemness" characteristics.

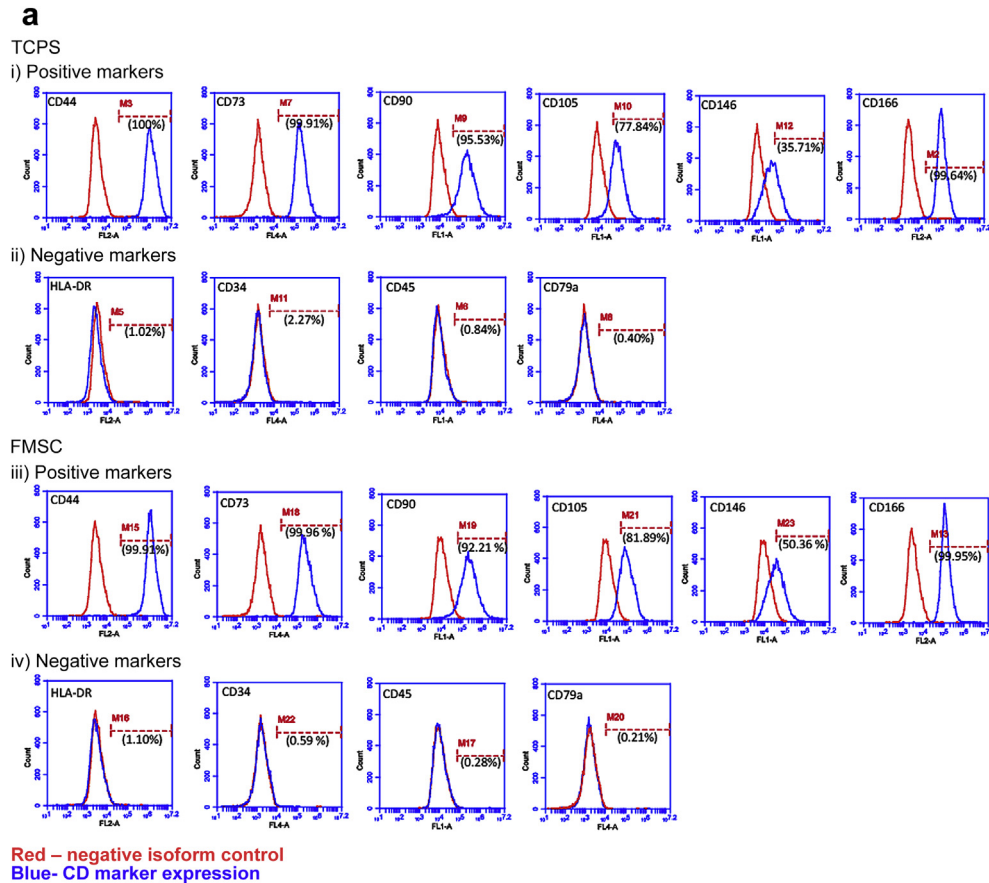
In addition in a separate screening, we investigated the ECM of neonatal fibroblasts (nHDF) for the expansion of aMSCs in comparison to TCPS (Supplementary Fig. S2). Fibroblast ECMs are of interest since they are one of the substrates used for expanding stem cells. While aMSCs proliferated more (about $1.7\times$ fold) on nHDF ECM compared to TCPS (Supplementary Fig. S2a), the cell size distribution for the aMSCs cultured on both substrates are similar however (Supplementary Fig. S2b). Furthermore, we tested some of the commercially available xenogenic-free substrates (Supplementary Fig. S3). However, the proliferation and size of the aMSCs on these substrates were not significantly improved and in some conditions, performed worse than our control TCPS substrates. Since the fibronectin coating, conditioned media conditions and xenogenic-free substrates did not improve or fared significantly worse than TCPS in terms of cell yield (Fig. 1d, $n = 3$, $p < 0.001$ and $p < 0.05$ for fibronectin and conditioned media respectively and Supplementary Fig. S2 for xenogenic-free substrates) and size (Table 1 and Supplementary Fig. S2), we did not include them in subsequent evaluations, which we focused on the ECM substrates.

3.2. Plating efficiency

All the ECM substrates (aMSC, fMSC and nHDF) were very effective in capturing the aMSCs within 2 h (Fig. 2), with plating efficiencies around 1 (Mean \pm S.D: 0.94 ± 0.09 , 1.07 ± 0.08 , 1.03 ± 0.06 for aMSC, fMSC and nHDF respectively, $n = 4$ for all). In contrast, the performance of glass substrates were significantly poor (0.27 ± 0.06 , $n = 6$) while TCPS were efficient (0.60 ± 0.06 , $n = 4$) but still significantly less than the ECM substrates ($p < 0.001$).

3.3. Flow cytometry analysis

hMSCs must express high levels of CD73, CD90, CD105 and lack expression of CD45, CD34, CD79a and HLA-DR, as defined by the

**b**

	AMSC ECM	FMSC ECM	nHDF ECM	TCPS
Positive Markers				
CD44	100	100	99.9	100
CD73	100	100	100	99.9
CD90	91.9	95.0	92.4	96.3
CD105	81.6	81.9	55.8	84.7
CD146	43.1	43.1	33.9	50.2
CD166	99.6	99.9	100	99.8
CD271	0.6	1.2	1	0.7
Negative Markers				
CD34	1.1	0.5	0.3	0.6
CD45	0.2	0.5	0.3	0.3
CD79a	0.2	0.4	0.3	0.7
HLA-DR	0.4	0.8	0.7	0.5

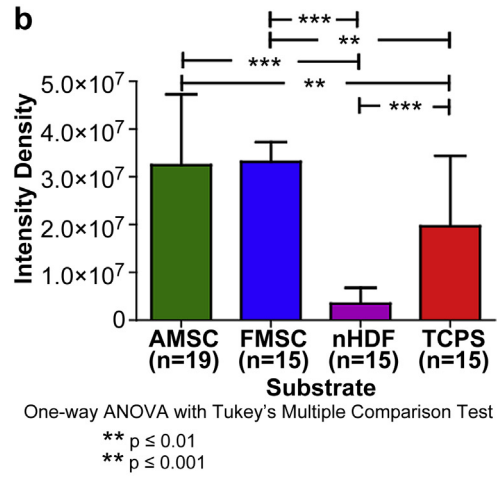
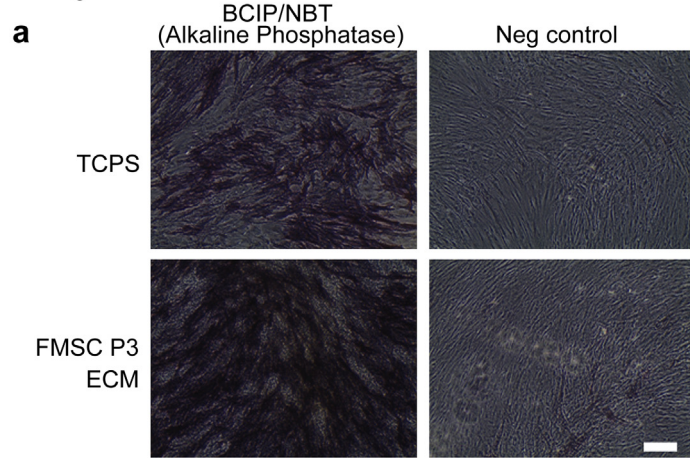
Fig. 3. Flow cytometry analysis of MSC markers for aMSCs cultured on TCPS (a-i and a-ii for MSC positive and negative markers respectively) and fMSC ECM (a-iii and a-iv for positive and negative markers respectively) with the particle count on the y-axes (0–800 in intervals of 200) and the fluorescence intensity data in terms of particle area (FL-A) on the x-axes (in log 10 scale from 10^1 to $10^{1.2}$) and (b) surface biomarker expression of aMSC cultured on various substrates.

Mesenchymal and Tissue Stem Cell Committee of the ISCT [16]. Our results in Fig. 3 shows that similar to their counterparts cultured on TCPS, aMSCs retain their immunophenotype when cultured on fMSC ECM.

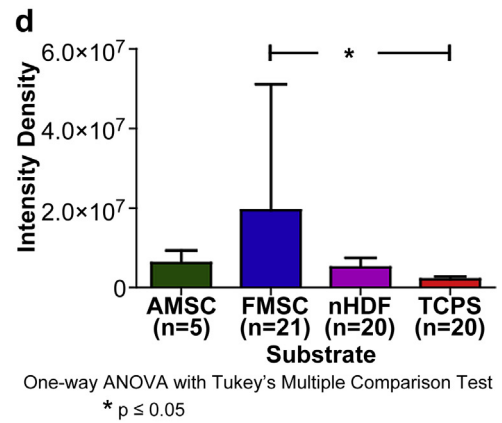
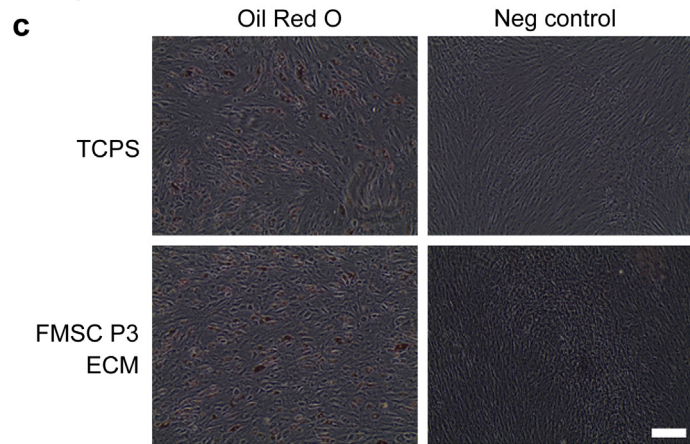
3.3. Differentiation assay

We have also demonstrated through *in vitro* assays that aMSCs plated on fMSC ECM are multi-potent as they are able to

Osteogenesis



Adipogenesis



Chondrogenesis

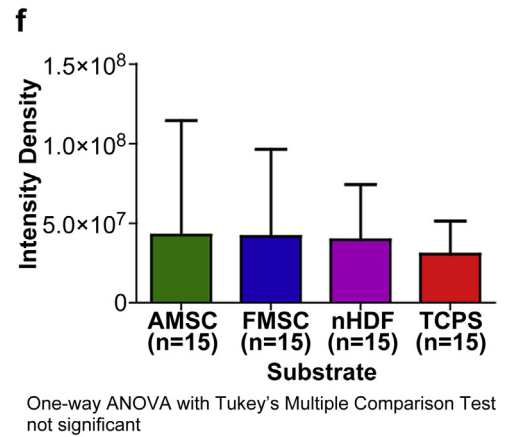
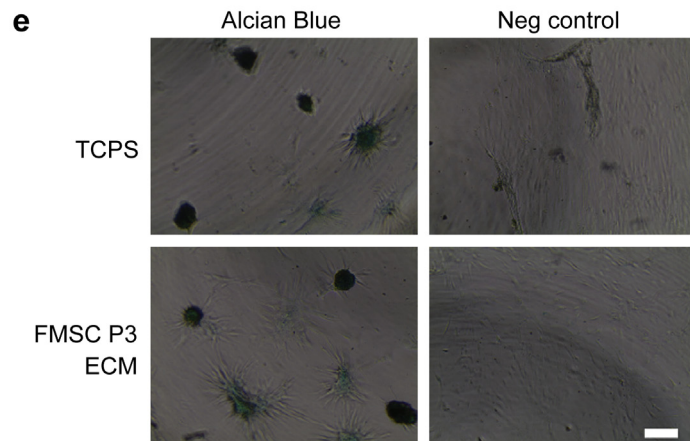


Fig. 4. Differentiation assays of aMSCs (P3 or P4) cultured on TCPS and fMSC ECM (a for osteogenesis, b for adipogenesis and c for chondrogenesis). For negative controls, aMSCs are cultured in growth media. The purple positive stain in the micrographs are of alkaline phosphatase expression after 14 days of selection pressure and evaluated using SIGMAFAST BCIP/NBT. Adipocytes are stained positively with Oil Red O and can be identified by their bright red color. Cartilage formation was detected with Alcian Blue, which stains aggrecan, an ECM component produced, dark blue. Quantification of differentiation expression levels of aMSC cultured on various substrates. The expression level is defined by the intensity density, as calculated by taking the product of the area and the mean of the stains in the images. Scale Bar = 200 μm.

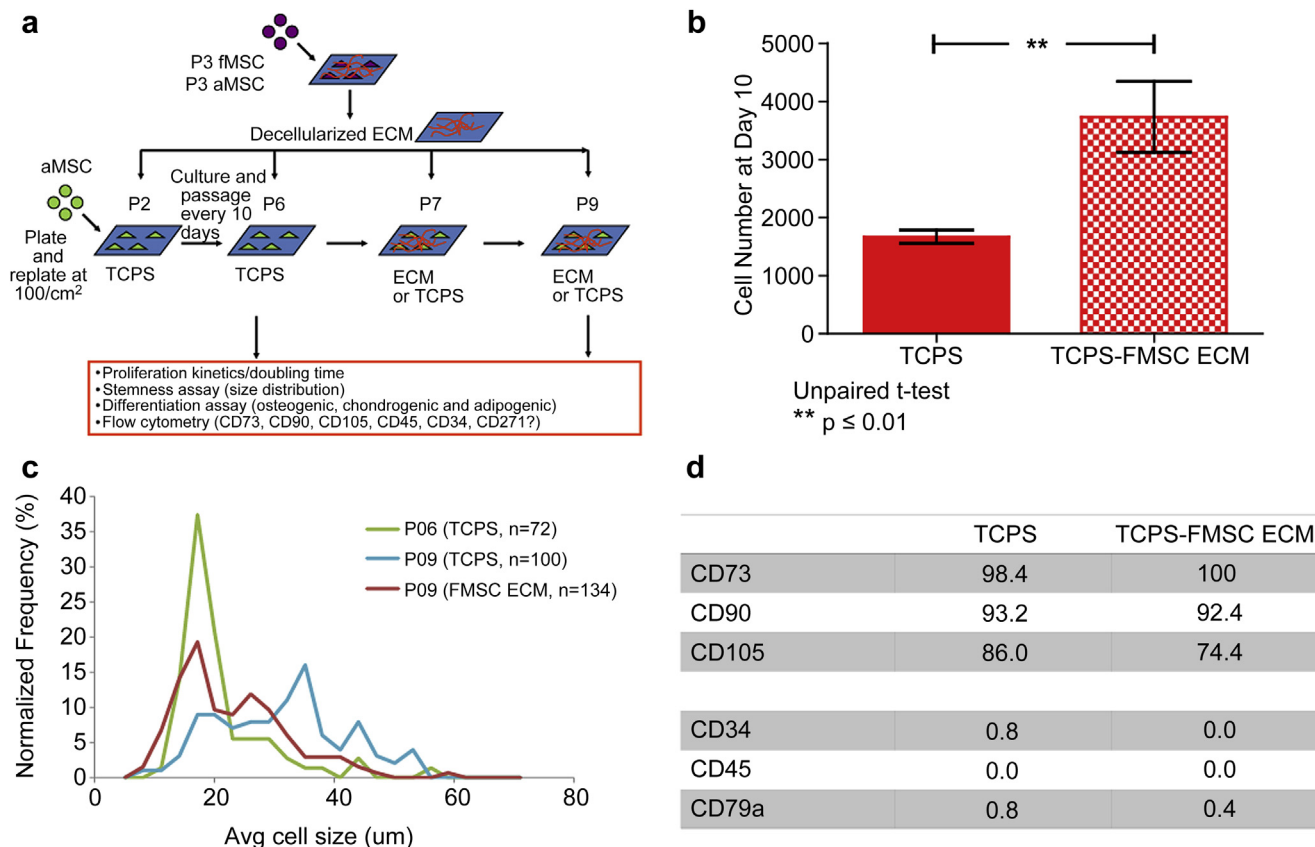


Fig. 5. (a) Schematic of late passage TCPS-cultured aMSC study, (b) Cell numbers ($n = 3$), (c) size distribution, and (d) surface biomarker expressions of late passage (P6) TCPS-cultured aMSCs that have been cultured under TCPS and FMSC ECM for 3 passages. Significant increase in cell proliferation observed as ascertained using 2-tailed unpaired t-test. ** $p \leq 0.01$.

differentiate into osteogenic, adipogenic and chondrogenic lineages based on positive alkaline phosphatase (AP) (purple stain in Fig. 4a), oil red o (red stain in Fig. 4c) and alcian blue (blue stain in Fig. 4e). The expression levels of the various stains in the images were quantified and presented as intensity densities (Fig. 4b, d and f). For osteogenic differentiation, the AP expression levels of aMSCs differentiated on aMSC ECM and FMSC ECMs were significantly higher ($p \leq 0.01$, $\sim 1.6\times$ and $\sim 1.7\times$ respectively, $n = 19$ and 15 respectively) than that of their controls on TCPS ($n = 15$) (Fig. 4b). Interestingly, the AP levels for aMSC on nHDF ECMs were significantly poor compared to the other conditions ($p \leq 0.001$, $\sim 9.3\times$, $9.5\times$ and $5.7\times$ lower to aMSC ECM, FMSC ECM and TCPS respectively, $n = 15$). For adipogenic differentiation (Fig. 4d), only the Oil Red O expression levels of the cells differentiated on FMSC ECM were significantly higher to their TCPS counterparts ($p \leq 0.05$, $\sim 9.3\times$, $n = 15$ for both). The average expression levels of aMSCs on the other ECMs were higher ($\sim 3.0\times$ and $\sim 2.4\times$ for aMSC ECM and nHDF ECM respectively, $n = 15$ for all) but not significantly different to that of TCPS ($p > 0.05$), suggesting that adipogenic differentiation potential of aMSCs were better retained when cultured on ECM substrates. Furthermore, the average Alcian blue expression levels for all ECM conditions were similar and higher than the TCPS

($\sim 1.4\times$, $\sim 1.4\times$ and $\sim 1.3\times$ for aMSC ECM, FMSC ECM and nHDF ECM respectively) but they were not significantly different ($p > 0.05$, $n = 5$, 21 , 20 and 20 for aMSC ECM, FMSC ECM, nHDF ECM and TCPS respectively) (Fig. 4f). The quantitative results here agreed with our qualitative observations that multi-potency of the aMSCs are better retained on FMSC ECM in contrast to their counterparts on TCPS and aMSC ECM. They also suggested that the FMSC ECM might be a superior, if not comparable, platform to TCPS for the culture of aMSCs.

3.4. Effect of plating late-passaged TCPS-cultured cells on FMSC ECM

To elucidate if late-passaged cells can be “rejuvenated” by FMSC-derived ECM, we took aMSCs that had been cultured on TCPS for 6 passages and plated them on FMSC ECM. After 3 passages, we assessed their cell yield, cell size and osteogenic potential in comparison to their TCPS counterpart (Fig. 5a). We observed significantly higher cell numbers (about 2.2-fold, $p \leq 0.01$, $n = 3$) compared to TCPS controls (Fig. 5b). In addition, culturing late passage aMSCs on FMSC ECM (labeled TCPS-FMSC ECM in the figure) retarded but did not prevent their gradual increase in cell

Table 1

Size distribution characteristics of aMSCs cultured on the various substrates and conditioned media (CM).

	aMSC P3 ECM	TCPS	Fibronectin	fMSC P2 CM	fMSC P3 ECM	fMSC P4 ECM
Median (μm)	17.2	17.5	22.4	22.5	15.5	18.5
25–75% percentile (μm)	15.7–20.7	15.8–20.2	17.9–29.4	20.0–26.5	13.9–18.0	15.3–18.5
Interquartile range (IQR) (μm)	5.0	4.4	11.5	6.5	4.1	5.3

size and reduction in uniformity (Fig. 5c). The cell size distribution for TCPS-FMSC ECM-cultured cells (P9) increased to $19.6 \pm 11.9 \mu\text{m}$ (Median \pm IQR) in contrast to the size distribution of the starting passage (P6, $16.8 \pm 5.9 \mu\text{m}$) (Table 2). In comparison, the TCPS-cultured cells (P9) had a higher median and wider distribution of $30.2 \pm 14.5 \mu\text{m}$. Furthermore, higher frequency of TCPS-FMSC ECM cultured cells (about 20% compared to about 10% for TCPS-cultured cells) was observed to possess similar cell size distribution in the 5–20 μm range compared to the starting passage (P6, green, about 40% in Fig. 5c). Main positive and negative biomarkers were screened for the 2 culture conditions (Fig. 5d) and their expressions were comparably similar. One exception was that CD105 expression of late-passaged TCPS-FMSC cultured aMSCs were lower than that of TCPS cultured aMSCs (74.4% vs 86.0%).

Interestingly, TCPS-FMSC ECM cultured cells improved their differentiation potential in contrast to TCPS-cultured cells as evidenced by the positive stain expressions after application of differentiation media (Fig. 6a). In addition, their average expression levels were higher compared to the TCPS-cultured counterparts. Quantification of the AP levels using SIGMAFAST™ BCIP NBT for TCPS-FMSC ECM cultured cells showed higher but not significantly different expression ($\sim 1.6\times$, $p > 0.05$, $n = 9$) to the TCPS-cultured controls (Fig. 6b). Oil Red O intensity densities were significantly higher for adipogenic differentiated TCPS-FMSC ECM cultured cells compared to TCPS-cultured cells ($\sim 2.1\times$, $p \leq 0.05$, $n = 7$ and 11 respectively). Furthermore, we found that there was a trend towards higher but not significantly different intensity densities of chondrogenic (Alcian Blue) expression for TCPS-FMSC ECM cultured cells in comparison to the TCPS-cultured cells ($\sim 18.7\times$, $p > 0.05$, $n = 5$ and 6 respectively). In addition, virtually all TCPS-cultured cells lost their chondrogenic potential as indicated by their Alcian Blue expression (Fig. 6a and b). Taken together, the study with late-passaged TCPS-cultured cells suggested the ability of the fMSC ECM to retard the senescence and rejuvenate the “stemness” of expanded aMSCs.

3.5. Quantification of ECM yield

Lastly to understand the scalability potential of the substrate for large-scale expansion of aMSCs for therapeutic applications, we quantified the amount of ECM produced per cell plated. We obtained $(0.5 \pm 0.2) \mu\text{g}$, $(1.4 \pm 0.6) \mu\text{g}$ and $(0.6 \pm 0.5) \mu\text{g}$ per cell plated for aMSC ($n = 6$), fMSC ($n = 4$) and nHDF ($n = 6$) respectively (Fig. 7). The ECM yield for fMSC is significantly higher than aMSC ($p \leq 0.05$). In addition, the yield for fMSC was also higher to that of nHDF but not significantly different ($p > 0.05$).

4. Discussion

Traditionally, MSC cells are expanded on tissue culture polystyrene (TCPS). However, they lose their regenerative capabilities after a few passages. Furthermore, results vary depending on the quality and brand of tissue culture polystyrene used [17]. To overcome these shortfalls, others have deposited individual ECM proteins such as fibronectin, collagen or laminin on the plastic substrates to culture and expand aMSCs. Recent alternatives are the

development of xenogenic-free substrates, which includes synthetic polymers or/and recombinant human matrices or matrix isoforms [2,3,18,19]. Many have become commercially available in recent years or are in the process of development. One of the drawbacks in growing cells *in vitro* using conventional tissue culture techniques and other above mentioned substrates is that these culture environments do not mimic their natural biological support enough or adequately in the physiological relevant manner. This natural support is a complex network of numerous macromolecules known as the extracellular matrix or ECM. The ECM holds cells and tissues together and provides a highly organized lattice within which cells can migrate and interact with each other. The matrix also plays an active and complex role in regulating the behavior of cells that are in contact with it, influencing their shape, migration, proliferation and metabolic functions [20,21]. Some of the ECM proteins found in the MSC ECMs are Collagen Type I, Collagen Type III, Fibronectin, Biglycan, Decorin, Perlecan and Laminin [9]. In contrast, cells grown on plastic and isolated proteins lose many of their natural multipotent properties since this approach fails to accurately mimic the complex protein interaction with the endogenous ECM, an environment that can be efficiently generated by a number of cellular populations.

The design of niche-like cell culture interfaces that maintain cells in their native behavioral state or instruct their phenotypic transformation towards that of a desired tissue may be the key to expanding MSC effectively. It has been previously demonstrated that cells cultured on ECM adopt growth characteristics, morphological appearance and biological responses which were not expressed when maintained on artificial plastic or glass substrata. This was observed even if the same substrates were coated with isolated constituents of ECM such as purified collagen or glycoproteins [22–26]. In recent years, there were studies that employed decellularized matrices from epithelial cells, endothelial cells, fibroblasts and adult bone marrow mesenchymal stem cells to expand the MSCs [8,9,14,27,28]. These studies concluded that decellularized matrices offer superior platforms to proliferate the cells in contrast to traditional methods. The use of naturally produced ECM, may also assure that the various matrix components will be found in their natural configuration and proportion. In this report, we investigated employing employing fMSCs, the most primitive and generative versions of MSCs, to produce the matrices for culturing the adult mesenchymal stem cells. Being similar cell types (except this is the fetal version), we hypothesized that their microenvironment would be the most similar, and therefore the most suitable for culturing these cell types. Furthermore, the process of decellularization destroyed the cells used to produce the matrices. Thus if autologous adult mesenchymal stem cells were used to generate the ECM, a large portion of the cells would be destroyed during the process resulting in the sacrifice of the already limited aMSCs. An alternative would be to rely on allogeneic sources for preparing the substrates. However, aMSCs suffered from more inconsistency since proliferation capacity and multipotency depended on the age/health of the donor. Furthermore, fMSC had been demonstrated to possess higher proliferative and osteogenic capacity compared to aMSCs and MSCs from other sources [11]. This implied that their ECM might be a superior substrate to expand and perhaps regenerate the stem cell behavior of adult cells. In addition, we might be able to generate larger quantities of ECM due to their superior growth. We observed here that only the decellularized fMSC matrices had shown significant increase in numbers of aMSC compared to TCPS during expansion. In contrast to previous literature [8,9], we did not observed increased proliferation on aMSC ECM in comparison to TCPS. It was possible that the TCPS substrates employed in our studies were more conducive for the expansion of aMSCs compared to those

Table 2
Size distribution characteristics of late passage TCPS-cultured aMSCs cultured on FMSC ECM in comparison to TCPS.

	TCPS (P6) – starting passage	TCPS (P9)	TCPS-FMSC ECM (P9)
Median (μm)	16.8	30.2	19.6
25–75% percentile (μm)	14.5–20.4	21.3–35.7	14.5–26.3
Interquartile range (IQR) (μm)	5.9	14.5	11.9

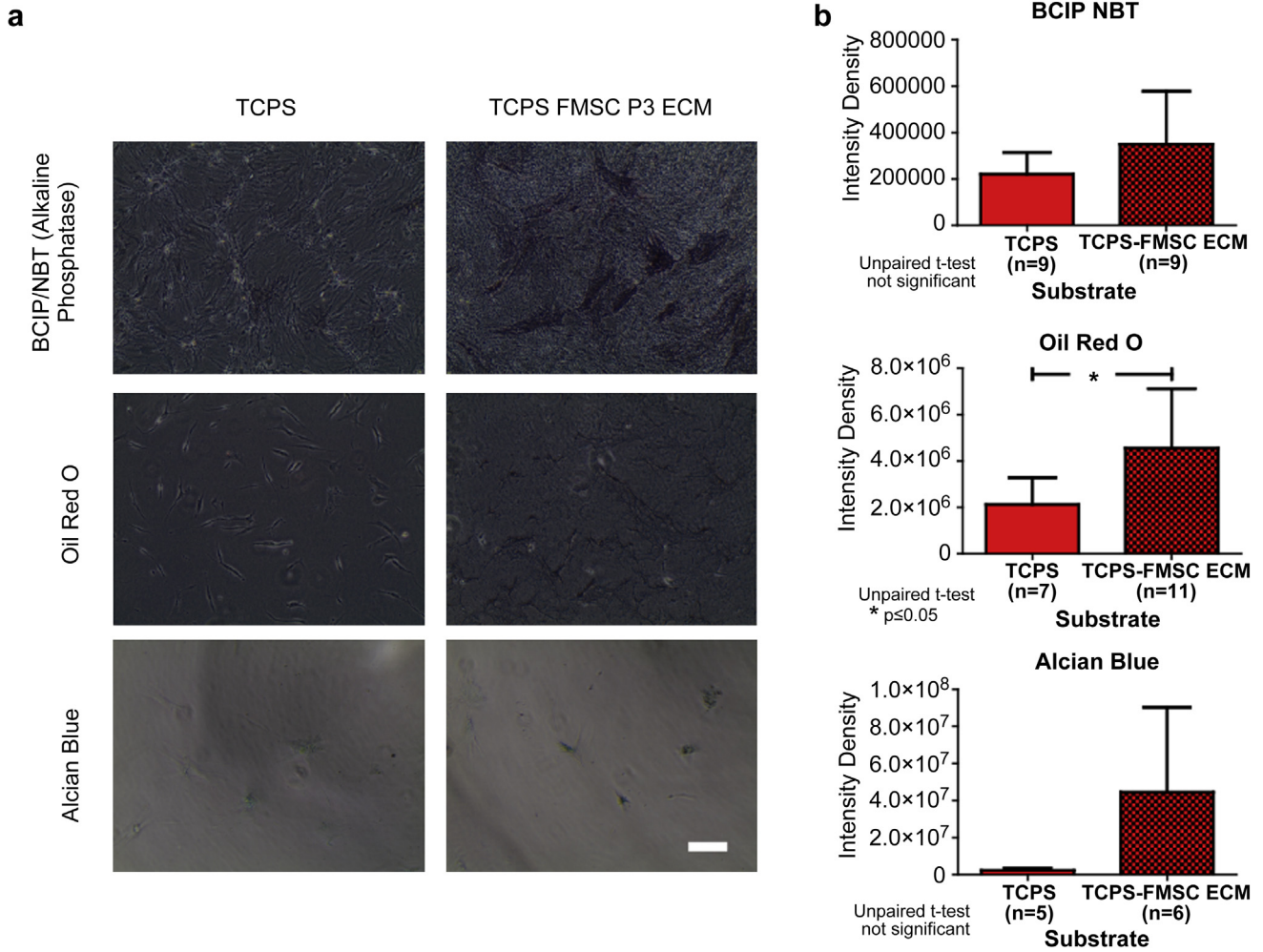


Fig. 6. (a) Representative differentiation micrographs and (b) expression levels of late passage (P6) TCPS-cultured aMSCs that have been cultured under TCPS and FMSC ECM for 3 passages. The purple positive stain in the micrographs are of alkaline phosphatase expression after 14 days of selection pressure and evaluated using SIGMAFAST BCIP/NBT. Adipocytes are stained positively with Oil Red O and can be identified by their bright red color. Cartilage formation was detected with Alcian Blue, which stains aggrecan, an ECM component produced, dark blue. The expression level is defined by the intensity density, as calculated by taking the product of the area and the mean of the stains in the images. Scale bar = 200 μ m.

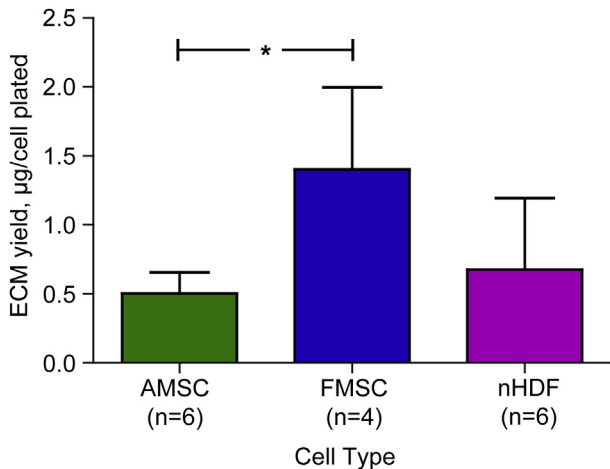


Fig. 7. ECM yield per cell plated. Statistical analysis using 1-way ANOVA with Tukey's Multiple Comparison Test; * $p \leq 0.05$.

used by others in previous literature; studies have shown that aMSCs plated on TCPS of various brands could vastly differ in their isolation, expansion and characteristics [17,29]. Alternatively, the aMSCs used in our studies might not be as potent (which according to the manufacturer, our lot was isolated from a 43-year old male donor and estimated to had undergone 7–8 population doublings by passage 2; a further 7 doublings before they were used to generate the substrates) as their equivalents used in previous studies. Interestingly as well, we obtained higher ECM yield from fMSC compared to the other cell types (aMSCs and nHDFs) (Fig. 7); this finding might explain why we experienced higher cell yield when aMSCs were expanded on ECM generated from the fMSCs as the increased ECM yield might contain higher amount of sequestered growth factors and provide a more conducive environment for proliferation. Our results thus suggested that fMSC ECMs as promising substrates to improve the *ex vivo* expansion of aMSCs for potential autologous applications such as clinical transplantation. However, this needs to be further validated through several different fMSC lines in order to reaffirm and improve the power of our observations. In addition, further studies as described below will be necessary to optimize the ECM substrates for translational applications.

Since the studies here were performed on mid-passages cells, one of the future studies is to culture the cells from the point of isolation where mononuclear cells from fresh bone marrow aspirates to their senescence to elucidate the long term effectiveness of the ECM substrates on expansion and maintenance of their progeny. We had performed a long-term study where we cultured and tracked the cell yield over 5 passages until the cultured aMSCs reached senescence. Commercially available cryopreserved purified aMSCs were plated directly on the various substrates and replated on the same kind of substrates after 10 days of expansion. We observed the loss of aMSC proliferation capacity and “stemness” in the later passages for all conditions and surprisingly, aMSCs reached senescence faster when cultured on fMSC ECM compared to TCPS and other substrates (data not shown). In addition in this study, long term culturing on fMSC ECM also did not maintain the multipotency of the aMSCs as we observed an increase in the cell size distribution. However, it was possible that our long term study was not conducted optimally. For example, we cultured them for 10 days before passaging; this usually resulted in almost confluent conditions (beyond the recommended 70%) for the aMSCs cultured on fMSC ECM in contrast to the other substrates due to its higher proliferative capacities in the early passages. As a result, this might have caused the cells to lose their proliferative potential and reach senescence faster in the later passages. To ascertain if this is the case, it will be interesting to repeat the long term studies but passage the cells at 70% confluency instead of every 10 days. Alternatively, the results may suggest that there are limitations to the life span of the aMSC population and culturing them under any of the tested ECM substrates will not extend their life spans. In addition, commercial available expanded aMSCs were used in the studies. Their preparation, expansion and cryopreservation methods may already committed some of the progenitors down certain lineages and may affect their subsequent fates on these substrates [30,31]. Indeed, we failed to detect one of the early positive biomarkers, particularly CD271 [32–35], in the early passages (Fig. 3).

Furthermore, our results (Fig. 1) indicated a passage-dependence on the quality of the ECM with the passage 3 ECM substrates performing better than ECM generated from Passage 4 in terms of cell yield and size. Therefore, we may to elucidate the effects of ECM produced by earlier passage (1 and 2) fMSCs. In addition, earlier passage cell populations contained contaminating cells from their isolation such as hematopoietic cells. The ECM of these contaminating cells might synergistically provide a more supportive environment on maintaining the multipotency of the MSCs. However, these series of studies may face difficulties to implement and may also not be feasible in terms of scaling up for applications due to limitations in their cell numbers.

Lastly, the ECM substrates were generated by culturing the cells to confluence and maintaining them under confluence conditions for one more week. This method from previous decellularization protocols for aMSC ECMs [8,9,14] were based on protocols from studies using epithelial and endothelial cells [36]. Culturing to confluence and beyond might not be ideal for MSCs since it was generally recommended to passage these cells at 70–80% confluence for optimal growth and prevent them from becoming senescent and lose pluripotency. The confluency of the MSCs might alter their phenotypes, and thereby changing the nature of the ECM they produced to one that is more committed towards certain lineages or provide cues for senescence. Therefore for it would be interesting to investigate the ECM generated during the growth phase. In addition, we noticed that the ECM may delaminate easily during the rinsing and treatment steps in our decellularization protocol; this was previously by others using similar decellularization protocols [37]. While we minimized the delamination through delicate

handling of our ECM culture wares, it may be necessary to improve the adhesion of the ECM to substrate through immobilization [37]. Alternatively, since the quantity produced may be limited, it might be necessary to develop transferable versions by retrieving and pooling the decellularized matrices to obtain sufficient amounts for testing. Nevertheless, our results overall represented the first steps towards the potential use of fMSC ECMs to improve the expansion and maintenance of aMSCs. To our knowledge, this is the first study in which matrices derived from BM fMSCs were generated and characterized for their expansion and differentiation potential. More studies, such as genetic stability characterizations, will be required to better understand the biological effects of aMSCs cultures under fMSC ECMs and translate the fMSC ECM substrate to expand aMSCs *ex vivo* for clinical applications.

5. Conclusions

In summary, fMSC ECM promotes aMSC expansion better than the other conditions including TCPS, aMSC ECM and nHDF ECMs in terms of superior growth, smaller and more uniform-sized cells. This phenomenon may be dependent on the age (passage number) of the cells producing the substrate. Late-passage aMSCs (previously cultured on TCPS for 5 passages) were significantly improved on the fMSC ECM platform in terms of proliferation, stemness and differentiation potential compared to continuous culture in standard TCPS conditions. We also quantified amount of ECM produced by each plated cell, which suggested feasible scalability for clinical applications. The results established fMSC matrices as promising platforms for *ex-vivo* expansion of human aMSCs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2014.01.081>.

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